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Structural requirements for dimerization, glycosylation, secretion, and biological function of VPF/VEGF

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Abstract

Vascular permeability factor (VPF) also known as vascular endothelial growth factor (VEGF), is a dimeric protein that affects endothelial cell (EC) and vascular functions including enhancement of microvascular permeability and stimulation of EC growth. To investigate the structural features of VPF/VEGF necessary for efficient dimerization, secretion, and biological activities, we employed site-directed mutagenesis with a Cos-1 cell expression system. Several cysteine residues essential for VPF dimerization were identified by mutation analysis of the Cys-25, Cys-56, and Cys-67 residues. Mutant VPF isoforms lacking either of these cysteines were secreted as monomers and were completely inactive in both vascular permeability and endothelial cell mitotic assays. VPF Cys-145 mutant protein was efficiently secreted as a glycosylated, dimeric polypeptide, but had a reduction in biological activities. The site of *N*-linked glycosylation was directly identified as Asn-74, which, when mutated produced an inefficiently secreted dimeric protein without post-translational glycosylation, yet maintained full vascular permeability activity. Finally, we found that one VPF mutant isoform Cys-101 was not secreted and this mutant functioned as a dominant-negative suppressor of wild-type VPF secretion as demonstrated by co-expression assays in Cos-1 cells.

Keywords: Angiogenesis; Cytokine; Growth factor; Mutant; Vascular permeability factor; (Endothelium)

1. Introduction

Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), consists of a family of polypeptide isoforms that specifically regulate endothelial cell function. VPF/VEGF has several demonstrated activities, including rapid enhancement of microvascular permeability in vivo [1–4], stimulation of endothelial cell growth [5–8], induction of transient uptake of calcium in endothelial cells [9], and promotion of angiogenesis [10,11]. VPF/VEGF mRNA and protein have been shown to be produced at high levels by human tumors as well as by transformed and tumor-derived cell lines [1,8,12–15]. The expression of VPF/VEGF mRNA has also been demonstrated in the developing embryo by in situ hybridization, suggesting that VPF/VEGF is one of the factors involved in coordinating vascular bed development and growth [16]. Moreover, VPF/VEGF expression is associated with the differentiation of mesoderm-derived embryonic cell lines into mature adipocyte and myotube phenotypes [17]. Thus, the VPF/VEGF family of proteins is likely to play a critical role in promoting angiogenesis associated with normal and pathological processes.

The VPF/VEGF polypeptides contain signal sequences for secretion [2,5,17] and are produced as disulfide linked dimers containing at least one glycosylation site in each polypeptide chain. Alternative splicing has been implicated in the formation of multiple human VPF/VEGF polypeptides consisting of 121, 165, 189, and 206 amino acids [18,19]. The predominantly expressed VPF_{165} polypeptide forms a glycosylated homodimer and has an apparent

Abbreviations: BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; flt, fems-like tyrosine kinase; flk-1, fetal liver kinase; GST, glutathione-*S*-transferase; HUVEC, human umbilical vein endothelial cells; KDR, kinase domain receptor; PDGF, platelet-derived growth factor; PDECGF, platelet-derived endothelial cell growth factor; TBS, Tris buffered saline; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

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molecular weight of 45 kDa as determined by non-reducing SDS-gel electrophoresis [20]. VPF and VEGF cDNAs isolated from mouse, rat and guinea pig encode 164 amino acids, one less amino acid than is observed in human or bovine species [17,21,22].

Two human tyrosine kinases, flt-1 [23] and KDR [24], have been identified as VPF/VEGF receptors. The mouse fetal liver kinase (flk-1) was shown to be the mouse homologue of the human KDR [25]. VPF/VEGF receptors belong to the class III tyrosine kinase receptor family, which bind dimeric ligands and include the receptors for PDGF, as well as the receptors for macrophage colony stimulating factor, and mast cell growth factor [26,27]. Expression of transcripts encoding the human flt-1 and KDR receptors have been localized to endothelial cells in tumor-associated vasculature [13,14]. The expression of the mouse flk-1 receptor, as measured by in situ hybridization, has also been found in endothelial cells during embryonic development [25].

The VPF/VEGF growth factors have low overall homology to the PDGF polypeptide family with particular conservation of the location of cysteine residues within both dimeric ligands [2,5]. The importance of particular cysteines in the activity of the PDGF-B homologue, v-sis, was demonstrated by mutation of specific cysteine residues, resulting in the loss of cellular transformation [28]. Structural studies have demonstrated that some of the cysteine mutations, which eliminate transforming activity, are involved in both intra- and intermolecular disulfide bonding [28,29]. To evaluate the features of the VPF/VEGF ligands required for function, particularly cysteine residues, we have utilized a Cos-1 cell expression system to produce normal and site-specific mutants of the mouse VPF protein. The mutations were performed at the cysteine amino acids with the intention of disrupting both inter- and intramolecular disulfide bonds. A potential *N*-linked glycosylation site was also mutated to determine directly whether this is a glycosylation sequence for VPF/VEGF. The information derived from these studies provide insights into the structural requirements for VPF dimerization, glycosylation, secretion, and functional activity. These studies also suggest additional means to suppress VPF/VEGF expression and function in other experimental systems.

2. Materials and methods

2.1. Vector construction and mutagenesis

SV40 replicating expression vector pMT2 [30] was obtained from Genetics Institute, Cambridge, MA. The mouse VPF/VEGF 164 amino-acid encoding cDNA (980 bp) [17] was cloned into pMT2 at the *Eco*RI cloning site and characterized for orientation. Mutagenesis was performed using single base mismatched oligos given below: Cys-25 (5′-CGAAGCTACAGCCGTCCA-3′), Cys-56 (5′-CTGATGCGCAGTGCAGGCTG-3′), Cys-67 (5′-GCCCTGGAGAGCGTGCCCAC-3′), Asn-74 (5′-GTCAGAGAGCTACATCACCATG-3′), Cys-101 (5′-CACAGCAGAAGTGAATGCAG-3′), Cys-145 (5′-GACTCGCGTAGCAAGGCGAG-3′). These oligos were used to mutate the wild-type mouse VPF cDNA using the oligonucleotide-directed in vitro mutagenesis system (Amersham). All mutants were characterized directly by double-strand DNA sequencing using the Sequenase kit (United States Biochemical) and confirmed in both 5′ and 3′ directions.

2.2. Cell culture, transfection, and media conditioning

SV40 transformed green monkey kidney cells, Cos-1, were obtained from American Type Culture Collection (ATCC) and maintained as described. Cells were cultured in complete Dulbecco's modified-Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (2 mM), and penicillin (10 U/ml)-streptomycin (10 μg/ml). Cells were plated at approx. 50% confluence. Medium was changed two hours prior to DNA transfection. DNA transfection was performed using the calcium phosphate precipitate method [31] with 10 μg of vector DNA per transfection. Wild-type vector DNA was normalized by addition of pBluescript SK$^+$ (Strategene) plasmid DNA. Calcium-phosphate/DNA precipitate was applied to cells and incubated for 6 h. Fresh DMEM was added and cells incubated for 16 h. Cell media was changed cells incubated for 24 h prior to analysis. Serum-free conditioned media was obtained by washing transfected or control cells 1 × with serum-free DMEM without phenol red, containing L-glutamine and penicillin-streptomycin. Cultures were then incubated with the same media at 7 ml per 100 mm^2 dish for 18 h at 37°C. The conditioned media was cleared of cells by centrifugation at 1000 × g for 5 min and frozen at −80°C.

2.3. Western blot analysis of conditioned media

Serum-free conditioned media obtained from cell incubations were subjected to reducing SDS-PAGE and blotted to nitrocellulose paper according to established procedures [31]. The nitrocellulose membrane was blocked with TBS containing 0.05% Tween-20 and 1% BSA for 30 min. The membrane was incubated at 4°C for 16 h with anti-rat N-terminal peptide antibody which preferentially detects reduced VPF amino acids 1–25 of rat and mouse amino acid sequence (22) (kindly provided by Dr. Janice Nagy, Beth Israel Hospital, Boston, MA.). The blot was washed 3 × in TBS-Tween 20 and visualization was performed with anti-rabbit IgG-Biotin and avidin-horse radish peroxidase using the Vectastain kit (Vector) using the conditions described by the manufacturer. The Western blot signals were then quantified by laser densitometry using an LKB Ultrascan XL densitometer.

2.4. Aortic endothelial cell mitotic assay

Bovine aortic endothelial cells (BAEC) were cultured in DMEM containing 20% FBS, L-glutamine, penicillin-streptomycin, and endothelial cell mitogen (Biomedical Technologies, Stoughton, MA). Primary cultures of BAEC were obtained as a kind gift of Dr. Dennis Lynch, Dana-Farber Cancer Institute, Boston, MA. BAEC cells were split once and frozen as early passage stocks (3 passages). Cells were seeded into multiple 6-well dishes at 4×10^5 cells/well and allowed to recover for 16 h in complete DMEM. The cells were then washed 2× with DMEM containing 5% FBS, L-glutamine and penicillin-streptomycin (5% FBS:DMEM). Control serum-free DMEM and cell conditioned DMEM were then added to 5% FBS:DMEM to a final concentration of 20% (v/v). Cultures were incubated with sample media at 48 h and harvested at 72 h. The cells, in triplicate, were harvested by trypsinization and counted 3× using a coulter counter (Coulter Electronics Model_Z_F). Total cell number was determined using the average of the three counts for each well analyzed. Three individual wells were used for graphic and statistical analysis.

2.5. Guinea pig microvascular permeability assay

Serum-free conditioned media obtained from transfection and control experiments was used directly in the Miles assay as described previously [1,4,32]. Increased vessel permeability was quantitated by spectrophotometric measurement of the Evan's blue dye extracted from skin test sites [33] and normalized using the mouse VPF sample as 100% activity.

2.6. Cellular labeling and immunoprecipitation

Cultures of control or transfected Cos-1 cells were placed in methionine and cysteine-free DMEM (ICN) containing L-glutamine, penicillin-streptomycin and [^{35}S]methionine/cysteine ExpreSS label (Dupont/NEN) at 200 μCi/ml, incubated for 4 h at 37°C and conditioned media collected and cleared by centrifugation at $1000 \times g$ for 5 min. The cell layer was washed 2× with PBS and collected with 1 ml lysis buffer (1% TX-100, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF). Cell extract was sonicated for 2 min, placed on ice for 10 min, and cleared by centrifugation at $13\,000 \times g$ for 10 min at 4°C. Samples were analyzed for ^{35}S-label incorporation into protein by trichloroacetic acid precipitation.

Immunoprecipitation and SDS-PAGE analysis was performed using equal counts of incorporated protein ($1 \cdot 10^6$ dpm per sample) as described for established procedures [31]. Antibodies utilized for immunoprecipitation were raised in rabbits to mouse VPF_{164}-glutathione-*S*-transferase fusion protein (anti-muVPF-GST). Antibodies were purified as IgG fractions by protein A-Sepharose affinity chro-

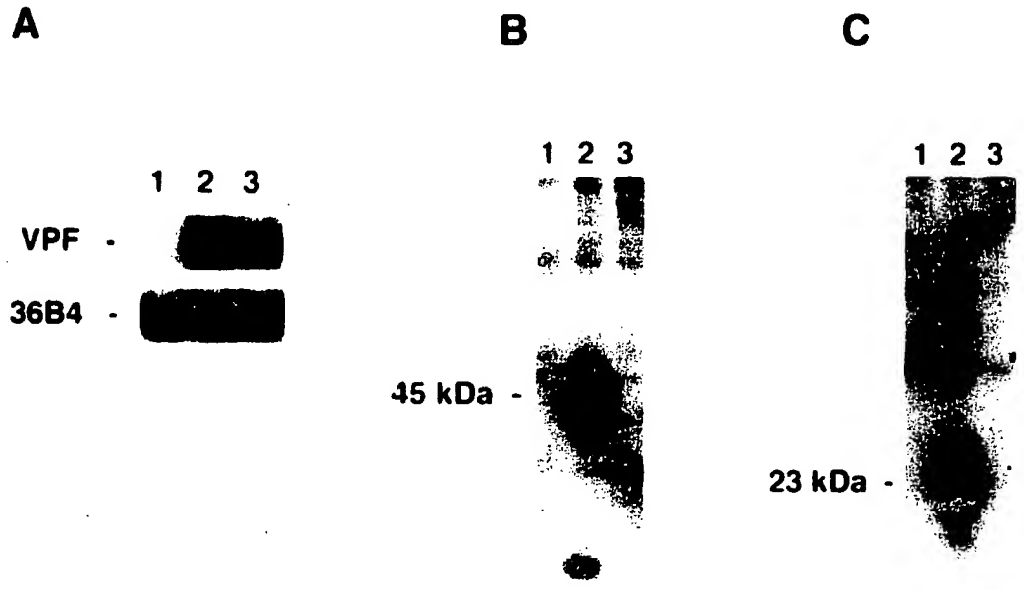

Fig. 1. Expression of recombinant mouse VPF/$VEGF_{164}$ by Cos-1 cells. Cos-1 cells were transfected with control pMT2 vector (lanes 1), VPF/$VEGF_{164}$ in the sense orientation (lanes 2), and VPF/$VEGF_{164}$ in the antisense orientation (lanes 3). Panel A, Northern blot hybridization of transfected Cos-1 cell RNAs probed for mouse VPF (VPF) and control ribosome-associated protein (36B4) mRNAs. Panel B, immunoprecipitation of ^{35}S-labeled secreted proteins from transfected Cos-1 cells with antibody to VPF; non-reducing SDS-PAGE analysis. Panel C, Same as panel B with reducing SDS-PAGE analysis.

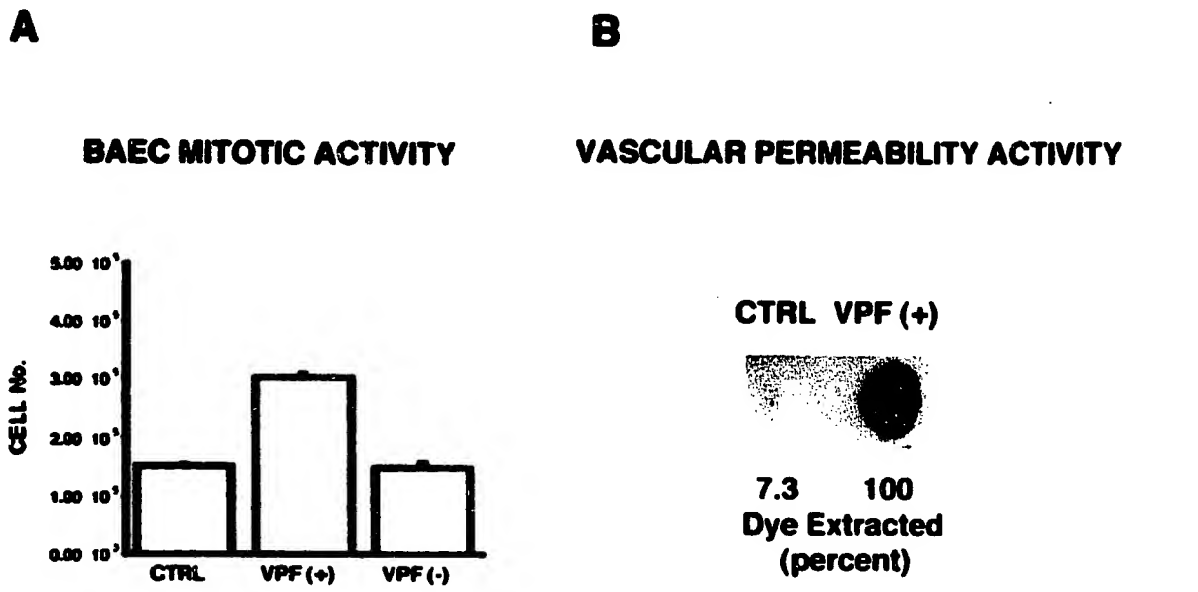

Fig. 2. Biological activities of Cos-1 expressed VPF/VEGF$_{164}$. Endothelial cell growth and microvascular permeability-enhancing activities present in serum-free conditioned media obtained from control pMT2 vector (CTRL), sense VPF/VEGF$_{164}$ (VPF(+)), and antisense VPF/VEGF$_{164}$ (VPF(−)) transfected cells. Panel A, Growth of BAECs over 4 days with a 1:10 dilution of conditioned media into control media. Cell number and standard deviation bars are given for assays done in triplicate for each sample. Panel B, Microvascular permeability-enhancing activity as determined in guinea pig dermal microvascular permeability assay. Serum-free conditioned media (0.25 ml) from pMT2 vector (CTRL) and sense VPF/VEGF$_{164}$ (VPF(+)) transfected Cos-1 cells was injected intradermally and the skin sites dissected and dye extracted as described in Section 2. Photographs of skin-test injection sites are shown and extracted dye units are expressed as relative percentage of VPF activity and indicated.

matography and used at a final concentration of 5 μg IgG/ml sample.

Total labeled conditioned media was analyzed by gel electrophoresis as follows: equal TCA precipitable counts (75000 dpm) of total labeled conditioned media was added to reducing SDS sample buffer and boiled for 5 min prior to electrophoresis in a 12.5% acrylamide-SDS gel. The gels were treated with Entensify (Dupont/NEN), dried, and exposed to Kodak X-OMAT film.

2.7. RNA isolation and Northern blot analysis

Total cellular RNA was isolated from cultured cell lines as described previously [34]. Northern blot analyses were performed using BioTrans nylon supported membranes (ICN) as described by the manufacturer. Mouse VPF$_{164}$ cDNA used for probe was the 980 bp fragment isolated and described previously [17]. A human ribosome associated protein, 36B4, cDNA probe [35] was used to control for RNA loading, blotting and hybridization. All cDNA probes used were isolated cDNA fragments which were radiolabeled with a random primed synthesis kit (Multi-Prime, Amersham). Blots were exposed to Kodak X-OMAT film. Densitometry was performed on sub-maximally exposed films using an LKB Ultrascan XL laser densitometer and the signal for VPF mRNA was normalized to the internal standard 36B4 hybridization signal.

3. Results

3.1. Expression of a functionally active recombinant mouse VPF / VEGF in Cos-1 cells.

Overexpression of VPF/VEGF for structural and biological analysis was accomplished by transient transfection of Cos-1 cells with the SV40 replicating vector, pMT2, containing the cDNA for mouse VPF$_{164}$. Fig. 1 demonstrates that these cells express both VPF mRNA and protein. VPF mRNA was detected in the cells transfected with the sense, VPF(+), and antisense, VPF(−), expression constructs (Fig. 1A). The secretion of VPF protein was determined by immunoprecipitation followed by non-reducing, SDS-PAGE analysis. Dimeric VPF protein was detected as a 45 kDa molecular weight species in non-reduced immunoprecipitated media samples from sense VPF(+) transfected cells only, but not in control, CTRL, or antisense, VPF(−), transfected cells (Fig. 1B). As expected, the VPF protein migrates as a 23 kDa band under reducing conditions (Fig. 1C). Therefore, the expression of VPF/VEGF$_{164}$ in this system is consistent with a dimeric and glycosylated structure for the 164 amino-acid polypeptide.

As described previously, the VPF/VEGF protein has been demonstrated to possess endothelial cell growth promoting [5–8] and microvascular permeability-enhancing

biological activities [1–4]. The expressed recombinant mouse VPF_{164} was therefore tested for both activities. As shown in Fig. 2A, a twofold increase in endothelial cell growth was demonstrated by supplementing media with conditioned media obtained from VPF(+) transfected Cos-1 cells, but not from the control or VPF(−) transfected medias. The mitotic activity observed in the VPF(+) transfected media was specific for endothelial cells as no detectable activity was observed when NIH3T3 fibroblast were used as target cells (data not shown).

To assay the permeability-enhancing activity of the recombinant $VPF/VEGF_{164}$ protein, we employed the microvascular permeability assay which measures the extravasation of serum protein-dye complexes into intradermal injection sites. The photograph of guinea pig microvascular permeability activity in conditioned media of cells transfected with control (CTRL) and sense VPF/VEGF (VPF(+)) vectors is shown in Fig. 2B. There was virtually no permeability enhancement in media obtained from control cells, whereas the media conditioned by VPF(+) transfected cells gives a substantial signal. Quantitation of dye extracted from injection sites demonstrated a ten fold higher signal with VPF(+) conditioned media than with control media. The signal to background ratio was substantially greater than that observed in the endothelial cell mitotic assay, which was only two- to threefold greater.

3.2. *Effect of amino-acid substitutions on VPF dimerization and secretion*

Mutations introduced into the mouse VPF cDNA and the resulting amino acid conversions are represented in Fig. 3. The majority of these mutations were designed to affect dimerization or protein folding by converting cysteine residues to serine amino acids, thus preventing possible disulfide bonds at these positions. These mutations were designated as C25-S, C56-S, C67-S, C101-S, and C145-S with the number indicating the position of cysteine residues replaced with serine. Also included in these mutational analyses was the alteration of the putative *N*-linked glycosylation site *asparagine*-isoleucine-threonine at amino acids 74–76 to *tyrosine*-isoleucine-threonine, indicated as A74-Y.

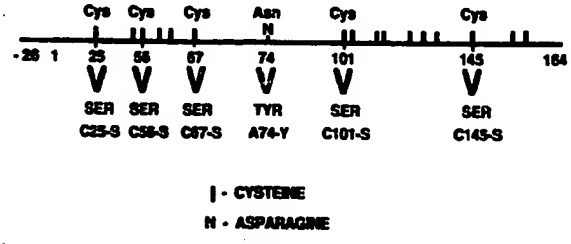

Fig. 3. Amino-acid conversions introduced into mouse $VPF/VEGF_{164}$ cDNA. Amino-acid residues in the wild-type polypeptide are given above the line drawing; amino-acid conversions resulting from mutations and the corresponding mutant designations are indicated below.

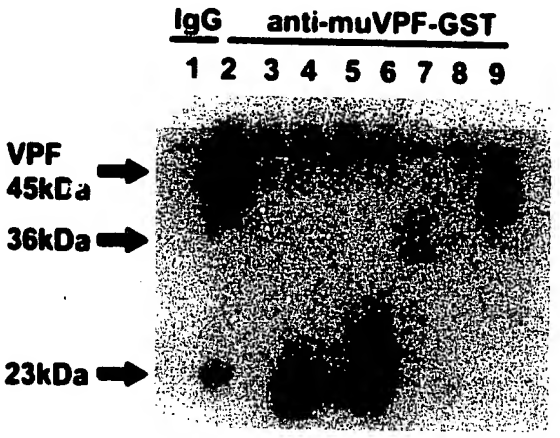

Fig. 4. Immunoprecipitation of wild-type and mutant $VPF/VEGF_{164}$ expressed by Cos-1 cells. Cos-1 cells were transfected with expression vectors for wild-type VPF, lanes 1 and 2, and mutant VPF isoforms, C25-S, C56-S, C67-S, A74-Y, C101-S, and C145-S, lanes 4–9, respectively. Metabolically ^{35}S-labeled conditioned medias were immunoprecipitated and analyzed with non-reducing SDS-PAGE as outlined in Section 2. Conditioned medias from transfected Cos-1 cells were immunoprecipitated with non-immune immunoglobulin G (IgG) or antibody to murine VPF (anti-muVPF-GST). Molecular weights of resulting bands are indicated on the left. The band at 50 kDa is non-specifically precipitated in all of the samples.

The pMT2 expression vectors containing the wild type and mutant VPF cDNAs were transfected into Cos-1 cells and the transfected cells were then analyzed for the production of VPF protein by immunoprecipitation with polyclonal antibody made to bacterial expressed recombinant mouse VPF-Glutathione transferase fusion protein (anti-muVPF). Metabolically labeled conditioned media ([^{35}S]methionine/cysteine) obtained from Cos-1 cells transfected with wild-type and mutant VPF expression vectors were immunoprecipitated with anti-muVPF and analyzed by non-reducing SDS-PAGE, Fig. 4. Dimeric VPF protein was observed only in the wild-type VPF, asparagine mutant A74-Y, and the cysteine mutant C145-S. Both wild-type and mutant C145-S showed appreciable expression levels of the 45 kDa dimeric polypeptide. However, the mutant A74-Y demonstrated an altered mobility species of 36 kDa, consistent with the predicted size of a non-glycosylated dimeric VPF protein.

Several mutations introduced into VPF/VEGF at cysteine amino acids, produced only monomeric polypeptides. Non-reducing SDS-PAGE analysis revealed monomeric polypeptides at 23 kDa with the cysteine mutants C25-S, C56-S, and C67-S. Thus, several mutant proteins were expressed as either dimers, A74-Y and C145-S, or monomers, C25-S, C56-S, and C67-S. In contrast, the cysteine mutant VPF, C101-S was not detected in conditioned media or in cell lysates (data not shown). To determine the relative levels of wild-type VPF expression in any particular transfection it was necessary to directly analyze wild-type and mutant VPF isoforms in conditioned

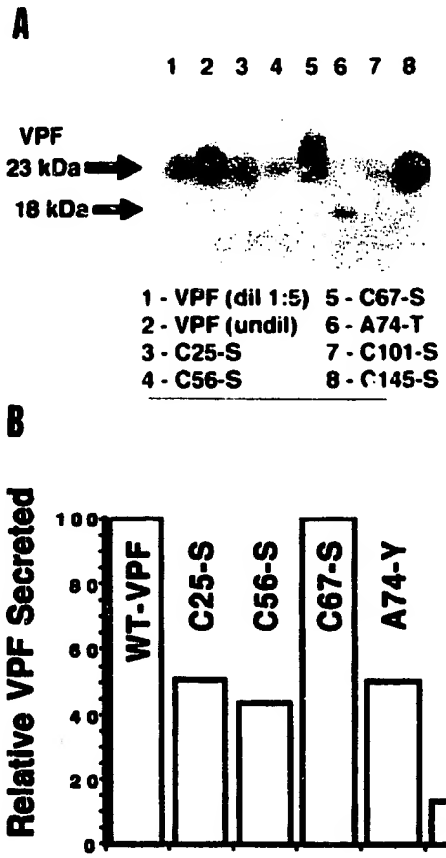

Fig. 5. Efficiency of wild-type and mutant VPF/VEGF$_{164}$ expression by transfected Cos-1 cells. Panel A, serum-free conditioned media obtained from equal number of transfected cells was electrophoresed in reducing SDS-PAGE, blotted and VPF polypeptides visualized with antibody raised to rat VPF N-terminal peptide. Conditioned media from wild-type VPF was either diluted 1:5, lane 1, or undiluted, lane 2. Conditioned medias from mutant VPF transfections were analyzed in undiluted form, lanes 3–8. Panel B, secreted wild-type and mutant VPF protein normalized to mRNA level. Wild-type and mutant VPF proteins detected in conditioned media by Western blot was normalized to mRNA levels as determined by Northern blot analysis. The relative protein production per mRNA was compared to wild-type VPF which was set at 100%. All mutant transfected cultures in this representative experiment produced mRNA levels equivalent or higher than the wild-type VPF transfected cells.

media. For this purpose Western blot analysis was performed using reducing SDS-PAGE, to eliminate differences in dimeric vs. monomeric proteins, and subsequent detection with antibody which recognizes mouse VPF/VEGF N-terminal peptide sequence. A typical Western blot analysis is shown in Fig. 5A. Since we have observed mutant VPF proteins that were expressed at both high and low levels, we compared their expression to diluted or undiluted wild-type VPF containing conditioned media, lanes 1 and 2, respectively. The precise amount of the mutant VPF proteins expressed was quite variable (lanes 3–8). The highest expression was observed in the C67-S and the C145-S samples. The altered mobility of the A74-Y mutant at 18 kDa is consistent with the predicted molecular weight of a 164 amino acid primary translation product without additional post-translational glycosylation, confirming that this is the actual glycosylation site for VPF/VEGF.

Although these data suggest that some of the mutant VPF isoforms are not synthesized and/or secreted with the same efficiency as the wild-type VPF, it was necessary to control for transfection efficiency. Therefore, duplicate transfections were performed to determine both protein and mRNA production in the same experiment. Conditioned media from transfected Cos-1 cells was obtained and quantified for wild-type and mutant VPF protein expression by direct Western blot/densitometry analysis. Total RNA from duplicate transfections were isolated and analyzed by Northern blot analysis for the level of wild-type and mutant VPF mRNA synthesized by the transfected vector. The hybridization signals were normalized to the signal obtained for the constitutively expressed mRNA for ribosome-associated protein, 36B4 [35]. Total VPF protein signal was divided by the total VPF mRNA signal to derive the relative amount of protein produced compared to steady-state levels of mRNA (Fig. 5B). Wild-type VPF protein was efficiently synthesized and secreted as was the mutant C67-S isoform, which was equivalent to wild-type, despite the fact that this isoform was clearly secreted as a monomeric polypeptide (see Fig. 4). The mutant VPF protein isoforms C25-S, C56-S, A74-Y, and C145-S were all produced at about 50% efficiency relative to wild-type VPF. In contrast, C101-S mutant VPF protein had approx. 10% of wild-type efficiency of production despite levels of mRNA expression equal to the wild-type VPF transfection. These combined data indicate that the C101-S mutant VPF protein is either inefficiently synthesized and secreted or is relatively unstable in Cos-1 cells, or both.

3.3. The C101-S VPF mutant isoform inhibits wild-type VPF synthesis

We investigated the possibility that mutant VPF proteins may function as dominant-negative suppressors of the wild-type protein when coexpressed in the same cell. In theory, dominant-negative inhibition of the wild-type VPF could occur by interference in translation, processing or secretion pathways. In addition, mutant wild type heterodimer formation may be targeted for rapid degradation or inhibit post-translational processing and secretion pathways.

As shown in Fig. 6, Cos-1 expressed VPF polypeptide was found to be detectable with reducing SDS-PAGE of total proteins secreted from the transfected cells. Also, as shown in Fig. 6, C101-S specifically interferes with the expression of wild-type VPF when co-transfected at a fivefold molar excess (lane 3 compared to lane 1). Co-transfection of C101-S with wild-type VPF resulted in a 95% reduction in vascular permeability-enhancing activity

as determined in duplicate transfections (data not shown) confirming the lack of VPF protein observed by SDS-PAGE analysis. Moreover, cotransfections of wild-type VPF with mutant C101-S resulted in no detectable VPF protein in cellular lysates, as determined by immunoprecipitation (data not shown). Lack of VPF accumulation in intracellular compartments indicates that the C101-S mutant either induces rapid degradation of a C101-S/VPF heterodimer and/or interferes with wild-type VPF synthesis directly. As demonstrated previously (Fig. 4), the C101-S mutant is not expressed at any appreciable level when transfected by itself (Fig. 6, lane 5). In contrast, cotransfection of wild-type VPF with C25-S does not significantly inhibit the synthesis of VPF polypeptide (Fig. 6, lane 2 compared to lane 1). Although the exact mechanism of C101-S suppression of wild-type VPF polypeptide is not clear, it appears to be a highly efficient process.

3.4. Comparison of functional activity between wild-type and mutant VPF proteins

Finally, the effect of mutant VPF/VEGF isoforms on functional activity was evaluated. Serum-free conditioned media obtained from wild type and mutant VPF expressing Cos-1 cells were directly quantitated by Western blot/densitometry analysis for total wild-type and mutant VPF proteins present. Following Western blot comparison of expressed wild-type and mutant VPF proteins, wild-type VPF conditioned media was normalized by dilution to

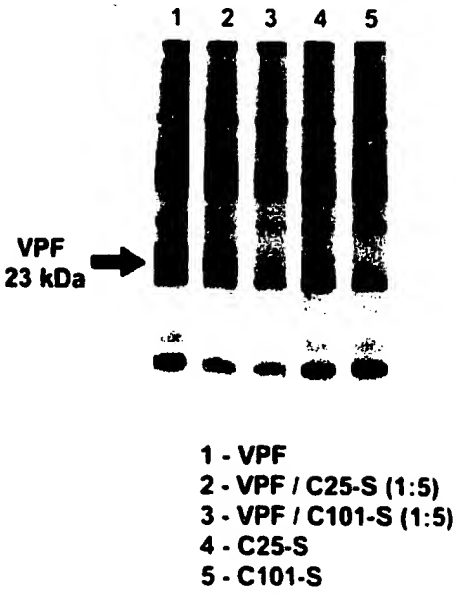

Fig. 6. Analysis of total secreted proteins in conditioned media from transfected Cos-1 cells. Total conditioned media from metabolically labeled cells were analyzed with reducing SDS-PAGE. Wild-type VPF was transfected alone, lane 1, or co-transfected with a fivefold molar excess of either C25-S or C101-S expression vector, lanes 2 and 3, respectively. Both mutant VPF expression vectors C25-S and C101-S were also used alone in transfections, lanes 4 and 5, respectively. The position of reduced VPF protein is indicated.

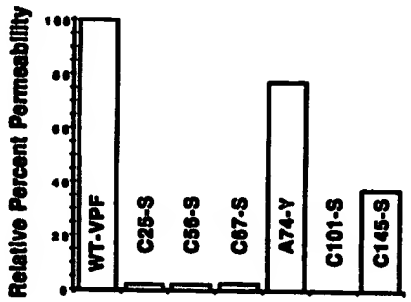

Fig. 7. Vascular permeability activity of wild-type and mutant VPF proteins. Panel A, vascular permeability was quantitated from duplicate injections and normalized to total reduced VPF protein detected by Western blot analysis of the same sample. Activity of wild-type VPF was set to 100% and the mutant isoform activities expressed as relative percentage of wild-type VPF.

equal the level of less efficiently secreted mutant VPF proteins. The normalized samples were then analyzed for VPF activity with the vessel permeability assay. The compiled data for VPF and mutant VPF microvascular permeability-enhancing activity are presented in Fig. 7. The results indicate that non-dimerizing single chain VPF mutants C25-S and C67-S have essentially no microvascular permeability-enhancing activity despite appreciable levels of protein production. Culture media from cells expressing VPF mutants C56-S and C101-S also did not possess any appreciable permeability-enhancing activity. The C145-S mutation, which was synthesized and secreted as a dimeric protein, demonstrated only partial permeability activity compared to the wild-type VPF. Multiple transfection and microvascular permeability analyses of this VPF/VEGF mutant demonstrated a consistently reduced activity level of 39.5 ± 5.0 percent ($n = 4$) when compared to wild-type VPF on a per molar basis. Since this mutation provided sufficient levels of dimeric protein expression, we also subjected the C145-S mutant protein to endothelial cell mitotic assays to determine whether this biological activity was similarly affected. We could not detect any enhancement of BAEC growth with this mutant when compared to the wild-type VPF (data not shown). Thus, although C145-S demonstrated some permeability-enhancing properties, no growth promoting activity was detected. Evaluation of the glycosylation mutation A74-Y in the vessel permeability assay revealed activity comparable to wild-type VPF at equal molar ratios, (Fig. 7). Therefore, glycosylation does not appear to be required for this biological activity. However, the A74-Y mutation greatly affected synthesis and secretion, since the protein production was reduced to about 50% wild-type secretion (Fig. 5B).

4. Discussion

We have investigated several structure/function relationships of the mouse VPF/$VEGF_{164}$ polypeptide. Cys-

teine residues essential for protein dimerization were identified as amino acids 25, 56, and 67. Mutation of these cysteines to serines eliminated the formation of dimeric protein resulting in secretion of monomeric VPF. These cysteine mutations which prevent the formation of dimeric VPF, are analogous to three of the four cysteines identified as essential for transforming activity of the PDGF homologue, v-sis (Cys-127, 160, and 171) [28]. The corresponding PDGF-B cysteines, Cys-16, 45, and 60, were found to be involved in intrachain disulfide bonding that formed the unique cysteine knot motif as determined by X-ray crystallography of the PDGF-BB homodimer [36]. Thus, both the VPF/VEGF and PDGF family of proteins are sensitive to several cysteine mutations for correct assembly to occur. Although the lack of protein dimerization observed in the VPF cysteine suggests that those residues form interchain disulfide bonds, it is also possible that they form intrachain bonds necessary for correct folding and/or alignment required for dimerization.

Mutations introduced at Cys-25, Cys-56, and Cys-67 prevent protein dimerization and yet the mutant isoforms were still secreted as monomeric polypeptides although at a lower rate than the wild-type VPF/VEGF protein. In our experience, harsh reducing, alkylating, and denaturing conditions are required for conversion of VPF homodimer to monomers and hence, these mutant monomeric VPF proteins provide a more suitable means to study the functional activity of the monomeric products. As measured by vascular permeability or endothelial cell mitotic assays, the expressed and secreted monomeric VPF polypeptides did not demonstrate biological activity, therefore, the dimeric structure of VPF/VEGF is required to elicit a biological response. Consistent with these findings, Class III receptor tyrosine kinases, similar to the VPF receptors flt-1 and KDR, are thought to require dimeric ligands to induced receptor dimerization and activation [29].

The Cys-145 mutant was efficiently glycosylated and secreted in dimeric form. Thus, unlike cysteine residues 25, 56, and 67, Cys-145 is not essential for dimerization. However, Cys-145 is essential for complete biological activity since both permeability and endothelial cell mitotic responses were affected by this mutation. The Cys-145 mutation demonstrated a reduced vascular permeability response of approx. 40% when compared to wild-type VPF, and no endothelial cell growth promoting activity was observed. It is possible that there are differential structural requirements for endothelial growth and vascular permeability. Alternatively, these observations may reflect the different quantitative sensitivities of the two assays employed or reduced stability of the Cys-145 mutant in the cell growth assay.

The primary amino acid structure of VPF/VEGF has a putative *N*-linked glycosylation site with the consensus sequence, asparagine-isoleucine-threonine, at amino acids 74–76. The asparagine-74 mutant protein proved to be a dimeric polypeptide with an altered electrophoretic mobility, consistent with lack of glycosylation. Reduction of this protein generated a polypeptide with an apparent molecular weight identical to the predicted size for a 164 amino-acid protein devoid of sugar residues. Therefore, this is the first direct evidence that the mouse VPF/VEGF Asp-74 residue is the *N*-linked glycosylation site. A major consequence of this asparagine mutation was a significant reduction in the secretion of the mutant VPF/VEGF isoform. There are many examples of a loss in secretion efficiency when glycosylation is inhibited. These include previous studies on VPF [37,38], as well as other proteins including erythropoietin [39], lipoprotein lipase [40], Von Willebrand factor [41], factor VIII [41], and tissue-type plasminogen activator [41]. Interestingly, the glycosylation-deficient VPF/VEGF polypeptide maintained complete vascular permeability-enhancing activity, which was indistinguishable from wild-type VPF on a per molar basis. These data are consistent with the observations of others who prevented VPF/VEGF glycosylation by tunicamycin treatment, yet the resulting protein still maintained activity [37]. Thus, the glycosylation at this site is likely to play little or no role in direct recognition of this ligand by VPF/VEGF receptors.

Finally, in the process of evaluating the key structural features of the VPF/VEGF protein required for function, we discovered a mutant isoform, C101-S, which is recognized by the cell, as an aberrant polypeptide, and is apparently degraded. The cysteine mutation at amino acid 101 prevented synthesis and/or secretion of this VPF isoform at any appreciable level. An analogous highly unstable mutation in PDGF-A has also been described [42]. Interestingly, this C101-S mutant VPF acts in a specific, dominant-negative fashion to limit the synthesis and/or secretion of coexpressed wild-type VPF. Thus, in mechanistic terms, the mutant VPF isoform efficiently targets the normal VPF/VEGF polypeptide for degradation. Presumably, this process occurs through an initial heterodimerization and subsequent destabilization of the mutant/wild-type dimer via active intracellular mechanisms. This dominant-negative mutant isoform could serve as an important tool for inhibiting VPF production in complex biological systems such as tumor growth and mammalian development.

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